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| 14. ABSTRACT The purpose of this study is to develop and characterize a surface plasmon resonance (SPR)-based assay that can specifically detect binding of APF to its cellular receptor, cytoskeleton associated protein 4 (CKAP4), immobilized on a sensor chip surface and to test the ability of this SPR-based assay to discriminate and measure the concentration of APF in urine from well-defined IC patients vs. age-matched, asymptomatic controls. In the second year of study, we have nearly completed our patient recruitment and urine sample collection. Testing of these samples by the cellular proliferation assay is underway at the University of Maryland. Further, considerable progress has been made toward the development, characterization, and eventual testing of clinical samples by the SPR assay. The focus in year two has been on development of the SPR assay using CKAP4 as a biosensor to detect APF. Our results demonstrate that we have successfully optimized rCKAP4 activity and immobilization with sufficient binding efficiency to detect and quantitate APF in a purified system. Importantly, we have determined that CKAP4 ₁₂₇₋₃₆₀ improves binding to APF with a normal proportional response to increasing dose of APF and the maximum binding response (Rmax). A parallel approach using an APF mAb as a biosensor was pursued to enhance sensitivity of the assay; it offers an alternate strategy to specifically measure APF in urine with the goal of developing a non-invasive, point-of-care diagnostic test for IC. | | | | | |
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1. INTRODUCTION:

Interstitial cystitis (IC) is a chronic, debilitating bladder disease that affects mostly women and is frequently misdiagnosed due to lack of a non-invasive test to detect the disease¹⁻⁶. While the cause remains unknown, biomarkers for IC have been described, including antiproliferative factor (APF), a glycopeptide that is detectable in the urine of 95-97% of IC patients vs. normal controls⁷⁻⁹. Validation of APF as a biomarker and etiologic agent for IC has been hindered by the absence of robust assays to detect and measure its concentration in patient urine. The purpose of this study is 1) to develop and characterize a surface plasmon resonance (SPR)-based assay that can specifically detect binding of APF to its cellular receptor, cytoskeleton associated protein 4 (CKAP4), immobilized on a sensor chip surface and 2) to test the ability of this SPR-based assay to discriminate and measure the concentration of APF in urine from well-defined IC patients vs. age-matched, asymptomatic controls. Approximately 90 patients will participate in this study. Urine specimens from 30 IC patients will be collected by Dr. Phillip Hanno and his staff during routine office visits to the Penn Urology IC Clinic at the University of Pennsylvania Hospital for the management of IC. Urine specimens from asymptomatic controls will be collected at TCMC by Betsy Mead, the clinical research coordinator. All processed urine samples will be shipped on dry ice to Dr. Susan Keay, who will blindly test the fresh urine specimens for APF activity by ³H-thymidine incorporation in addition to 30 banked frozen specimens (from another 15 IC female patients and 15 age-matched, asymptomatic controls) for comparison to results obtained from the SPR assay. We expect that the SPR-assay will overcome current barriers associated with validation of APF as a diagnostic biomarker for IC by being able to specifically detect the presence of APF in urine and accurately quantitate its levels for the first time. This would meet a critical need for an affirmative, diagnostic test for IC with the advantages of being rapid, specific, and non-invasive; further, it would present major learning opportunities for advancing our knowledge about the contribution of APF to IC.

2. KEYWORDS:

| | |
|-----------|---|
| APF: | antiproliferative factor |
| CKAP4: | cytoskeleton associated protein 4 |
| ED: | extracellular domain |
| FL-CKAP4: | full-length cytoskeleton associated protein 4 |
| HT-CKAP4: | histidine (x6) tagged-cytoskeleton associated protein 4 |
| IC: | interstitial cystitis |
| kDa | kilodalton |
| KLH: | keyhole limpet hemocyanin |
| mAb: | monoclonal antibody |
| NTA: | nitrilotriacetic acid |
| PAGE: | polyacrylamide gel electrophoresis |
| PBST: | phosphate buffered saline containing Tween-20 |
| SDS: | sodium dodecyl sulfate |
| SPR: | surface plasmon resonance |

3. ACCOMPLISHMENTS:

Major Project Goals

The original Statement of Work indicated two specific aims or major goals to be accomplished during the 3-year funding period, with emphasis on the first aim in the first two years and the second aim in the final year: 1) Develop and characterize a SPR-based assay employing a CKAP4 immobilized biosensor to detect APF (1-24 months) 2) Determine the ability of the SPR-based assay to detect APF in urine from patients with IC (1-36 months)

The status of milestones for tasks related to each aim of the project is summarized below.

Specific Aim 1:

Task 1a. Optimization of rCKAP4 activity and immobilization on sensor chip surface (months 1-12)

Milestone: *Improved CKAP4 binding efficiency to APF in a purified and non-purified system (ie, urine)*

Completion Date: Completed in a purified system December 2014; 90% complete in urine

Task 1b. Optimization of conditions for sensor chip surface regeneration and binding reproducibility (months 1-12):

Milestone: *Full regeneration and CKAP4/APF binding reproducibility following 3 repeated cycles of analyte and regeneration injections.*

Completion Date: Completed January 2015

Task 1c. Characterization of the SPR-based assay using synthetic APF (months 12-18):

Milestone: *Characterization of 20 samples containing various levels of synthetic APF spiked in urine from healthy donors as a first test of the system's diagnostic ability.*

Completion Date: 50% complete

Specific Aim 2:

Task 2a. Regulatory review and approval by Institutional Review Boards and DoD Human Research Protection Office (months 1-3):

Completion Date: Completed August 2013

Task 2b. Recruitment of human subjects for urine sample acquisition (months 4-24):

Milestone: *Acquisition of 60 urine specimens from 30 IC/PBS patients and 30 age-matched, asymptomatic controls.*

Completion Date: 99% complete

Task 2c. Testing of biological urine specimens by the cellular proliferation assay (months 4-30)

Milestone: *To have tested 90 urine specimens for APF activity using the cellular proliferation assay.*

Completion Date: 28% complete

Task 2d. Testing of biological urine specimens by the SPR-based assay and comparison of results with cellular proliferation assay results (months 24-36)

Milestone: *To have tested 90 urine specimens for APF using the SPR-assay.*

Completion Date: 0% complete

Task 2e. Statistical analysis (months 24-36)

Milestone: *Assessment of APF's utility as a diagnostic biomarker for IC and the first direct measurement of APF in human urine.*

Completion Date: 0% complete

Accomplishments in Year 2

We have predominantly focused our effort in the second year of the funding period on development and characterization of the SPR-based assay. Below we present the original SOWs (in italics) and our major accomplishments (underlined> over the second year.

SOW – Specific Aim 1/Task 1: *Develop and characterize a SPR-based assay employing a CKAP4 immobilized biosensor to detect APF.* During this annual reporting period, focused effort continued on Tasks 1a, 1b, and 1c under Specific Aim 1. We have made substantial progress on each task as detailed below.

1a: *Optimization of rCKAP4 activity and immobilization on sensor chip surface (months 1 -12).* In order to develop a robust method to immobilize CKAP4 onto a sensor chip surface to measure APF binding, optimization of recombinant CKAP4 (rCKAP4) activity was pursued and achieved. Various strategies were employed to promote CKAP4's most physiologically relevant conformation in order to obtain improved APF binding efficiency. Early results indicated that a truncated form of CKAP4 consisting of

its extracellular domain (ED) achieved stronger binding to APF than full-length forms tagged with Histidine at the N- or C-terminus. Therefore, we focused our efforts in year 2 on characterization of four CKAP4 deletion mutants of the extracellular region in an effort to identify the primary APF binding domain and thus establish a robust binding assay with much higher sensitivity.

Using structural prediction software, we analyzed the CKAP4 ED and determined the optimal deletion strategy for generating mutants of this region while preserving structural and functional domains. Four CKAP4 deletion mutant constructs consisting of amino acids 106-602, 127-360, 361-524, and 525-602 were generated by PCR and subcloned into the pET15b bacterial expression vector. All of the mutants (tagged at N-terminus with His₆) were expressed successfully in bacteria. Each mutant was successfully purified, dialyzed, and immobilized to a CM5 chip via amine-coupling to measure its APF binding response. As shown in Figure 1, the 127-360, 361-524, and 525-602 mutants exhibited specific binding to active APF, with mutant 127-306 exhibiting a slightly higher binding affinity and intensity. Table 1 lists the parameters of the binding kinetics for the APF interaction with the CKAP4 extracellular domain mutants using a 1:1 binding kinetics model. It is interesting to point out that both CKAP4₁₂₇₋₃₆₀ and CKAP4₃₆₁₋₅₂₄ exhibited a fast association constant (k_{on}) and a slow dissociation constant (k_{off}), therefore yielding high binding affinity ($K_D = k_{off}/k_{on}$; K_D for CKAP4₁₂₇₋₃₆₀: 1.34 μ M, and K_D for CKAP4₃₆₁₋₅₂₄: 2.27 μ M), which fits the general model of strong interaction and suggests that the primary APF binding site is located within the Aa 127-524 region of the CKAP4 extracellular domain. In contrast, interaction of APF with the CKAP4₅₂₅₋₆₀₂ ligand (Fig. 1C) displayed different binding kinetics with a slower association phase and a rapid dissociation phase, conveying a much lower affinity with a K_D value of 29.64 μ M. Although the 106-602 mutant also bound APF efficiently (data not shown), it did not show reproducible binding, suggesting that the presence of the receptor transmembrane domain requires an alternative immobilization strategy and sensor chip surface to preserve the structure of CKAP4 (ie, L1 chip). Thus, we determined that the CKAP4₁₂₇₋₃₆₀ and CKAP4₃₆₁₋₅₂₄ mutants exhibit improved binding to APF with normal proportional response to increasing dose of ligand and the maximum binding response (Rmax), making a robust SPR-based assay feasible.

Table 1. Kinetic parameters of the interaction between APF and CKAP4 ED deletion mutants

| Ligand | k_{on} | k_{off} | K_D | R_{max} |
|--------------------------|-----------------------------|-----------------------------|-------------------------|-----------------------------|
| | $M^{-1} s^{-1} \times 10^2$ | $s^{-1} \times 10^{-4}$ | μM | RU |
| CKAP4 ₁₂₇₋₃₆₀ | 3.57 | 4.78 | 1.34 | 60.49 |
| CKAP4 ₃₆₁₋₅₂₄ | 3.72 | 8.44 | 2.27 | 36.26 |
| CKAP4 ₅₂₅₋₆₀₂ | 1.77 | 52.5 | 29.64 | 27.36 |

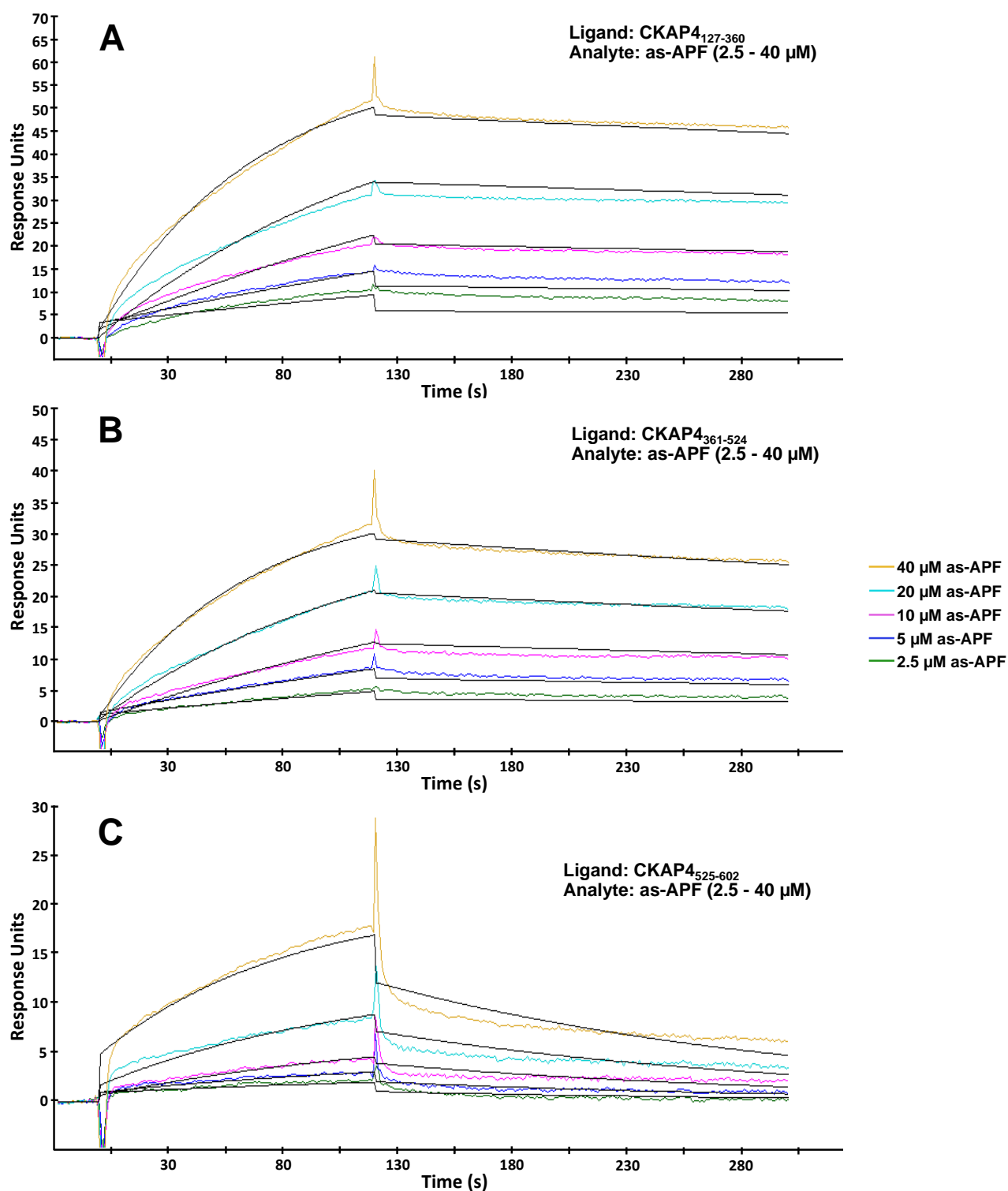


Figure 1. Binding kinetics of APF interaction with CKAP4 extracellular domain deletion mutants. Three CKAP4 ED deletion mutants were immobilized onto the Fc2 channel of CM5 chip as the ligand; the Fc1 channel was treated similarly but without the protein as the control. Multi-cycle kinetics assay were performed by the injection of various concentrations of as-APF (2.5 – 40 μ M, colored lines) over CM5 chip surfaces on which CKAP4₁₂₇₋₃₆₀ (**A**), CKAP4₃₆₁₋₅₂₄ (**B**), and CKAP4₅₂₅₋₆₀₂ (**C**) were immobilized via amine-coupling. The adjusted sensorgrams (Fc2-Fc1) were overlaid to calculate the binding kinetics. The 1:1 binding model was used to fit the data and fitting curves (black lines) are also shown. The parameters of binding kinetics are shown in Table 1.

Based our findings using CKAP4 variants, we sought to test an alternative, parallel approach that may significantly increase the sensitivity of our SPR assay. This methodology utilizes a monoclonal anti-APF antibody as the biosensor in the SPR assay to detect APF as the analyte. Using keyhole limpet hemocyanin (KLH)-conjugated APF on the surface of a CM5 chip, we screened 13 primary hybridoma clones (previously generated by our laboratory) for APF sensitivity using the SPR assay and identified 6 clones that demonstrate high binding affinity to APF. This result was confirmed by antibody purification, and two of these APF hybridomas (7E11 and 6E7) were selected for further subcloning based on their performance in ELISA and SPR analyses. During the final 6-well cloning stage, 12 clones from the 7E11 (IgG1, k chain) and 6E7 (IgG3, k chain) hybridomas were screened by ELISA, Octet, and SPR to determine their specificity for APF, their antibody quantitation, and their secretion. Two clones from the 7E11 hybridoma and three from the 6E7 hybridoma were selected for large-scale production and purification (Figure 2A). All five purified monoclonal antibodies displayed specific activity against APF-KLH in a dot blot assay (Figure 2B).

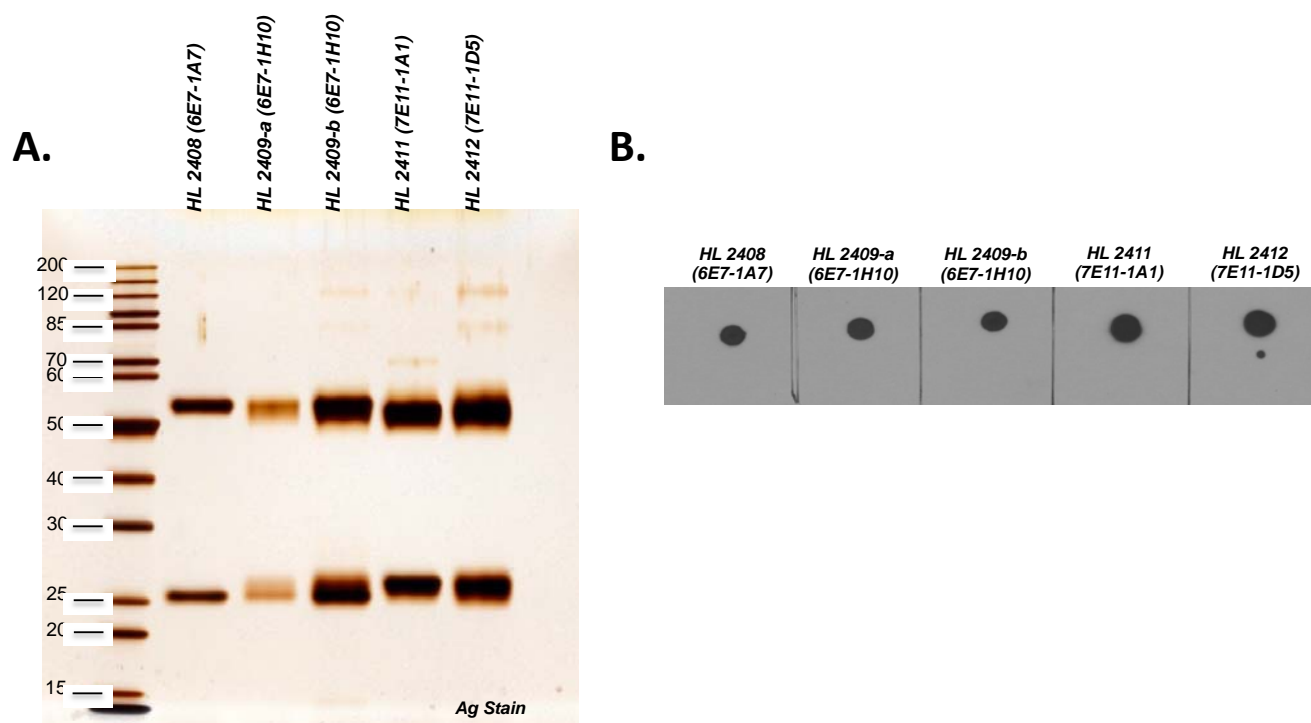


Figure 2. Confirmation of the affinity of final APF monoclonal antibodies after large-scale production and purification. **A.** Confirmation of purity for 5 antibodies selected for final large-scale production and purification. 1 µg of each mAb (based on UFL provided concentrations) was loaded on a 10% gel and stained with silver stain. **B.** Dot blot was performed as follows: 0.01 µg KLH-APF (in 5 µl PBS) was spotted on 5 separate nitrocellulose membrane strips and allowed to air dry. The membranes were then incubated in 5% non-fat dry milk (in TBS-T) for 1 hr at RT to block non-specific sites. Membranes were then separately incubated at 4 °C, overnight in purified mAb primary antibody solutions (0.5 µg/ml). Membranes were then washed with TBS-T, incubated in secondary antibody solution (1:2,000 dilution of our stock anti-Mouse IgG) for 1 hr at RT, and developed by ECL method.

In order to further characterize and test these mAbs for APF specificity by SPR, we investigated the optimal immobilization strategy for binding to the sensor chip surface (Figure 3). Using an amine-coupling approach, the APF monoclonal antibody (mAb), HL-2411, displayed specific binding against APF, as-APF (3A and 3B), KLH-APF (3C), but not APF lacking its sugar moieties (3D). We also compared and measured HL-2411 mAb binding to APF by SPR using an indirect capture immobilization approach as described in Figure 4. Again, HL-2411 displayed specific binding against KLH-APF and as-APF (4C and 4D), but not APF lacking its sugar moieties (4E); further, this binding was more enhanced, suggesting that the indirect capture immobilization approach presents the

antibody in a better conformation that can be recognized by APF. The four remaining monoclonal antibodies—HL-2408, HL-2409a, HL-2409b, and HL-2412—were also tested and all displayed specific binding against APF, as-APF, and KLH-APF albeit with varying sensitivities.

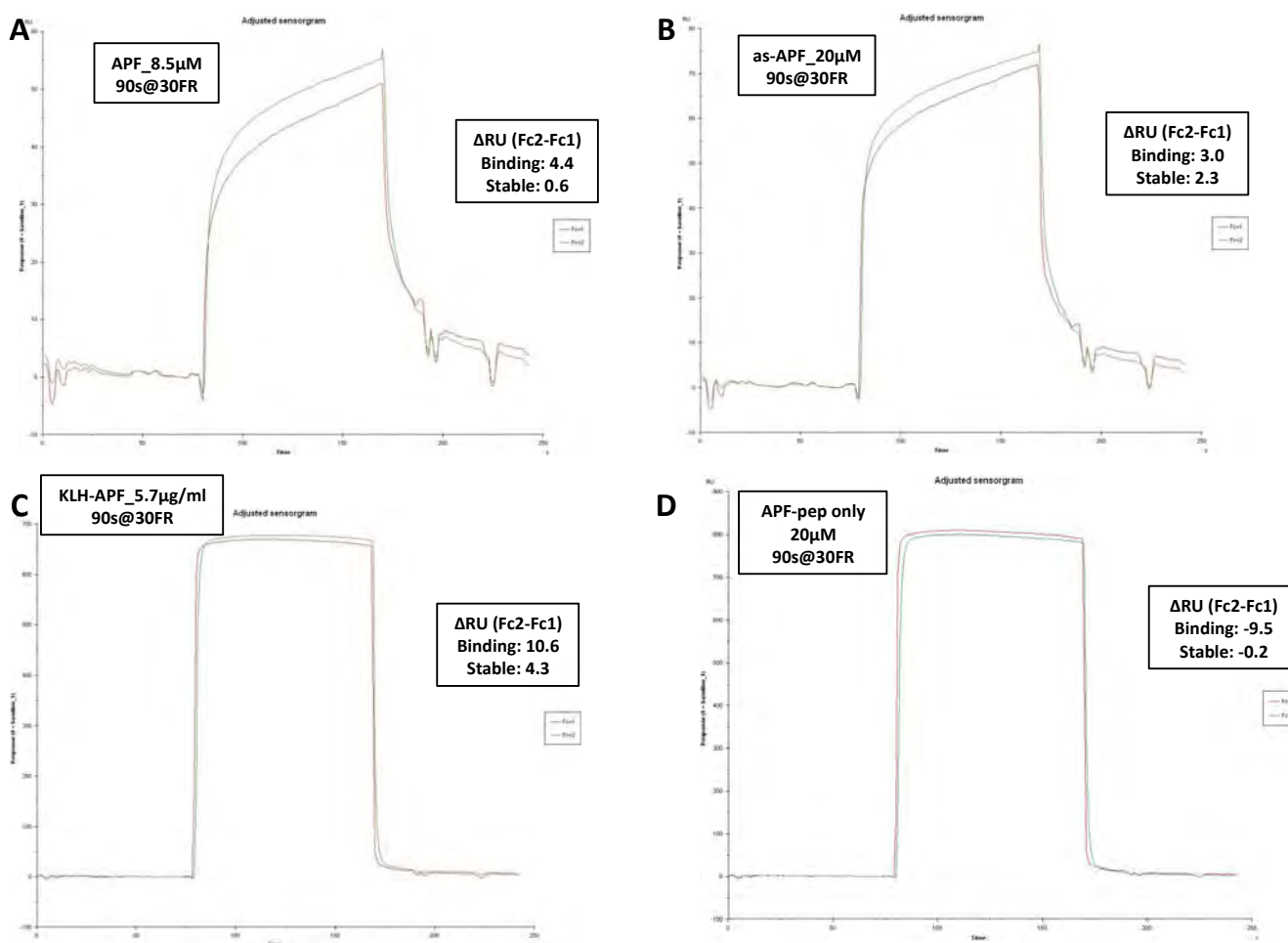


Figure 3. Measurement of APF monoclonal antibody (mAb) HL-2411 binding to APF by SPR through amine-coupling immobilization approach. APF mAb HL2411 was directly immobilized on Fc2 channel of a CM5 sensor chip by amine coupling. Fc1 channel was blocked using amine-coupling reagents. 1581 RU of HL2411 (Fc2-Fc1) was immobilized to Fc2 channel. HL-2411 mAb was assayed for binding against various APF derivatives at different concentrations and flow rates. Representative sensorgrams are shown as follows: APF as analyte (**A**), as-APF (asialo-APF) as analyte (**B**), KLH-APF (APF conjugated to keyhole limpet hemocyanin) as analyte (**C**), and APF peptide (lacking sugar moiety) as analyte (**D**). Analyte concentrations, flow rates (FR), contact times (90s), and Δ RU (Fc2-Fc1) are indicated on each panel. Interactions were measured on Biacore X-100 Plus.

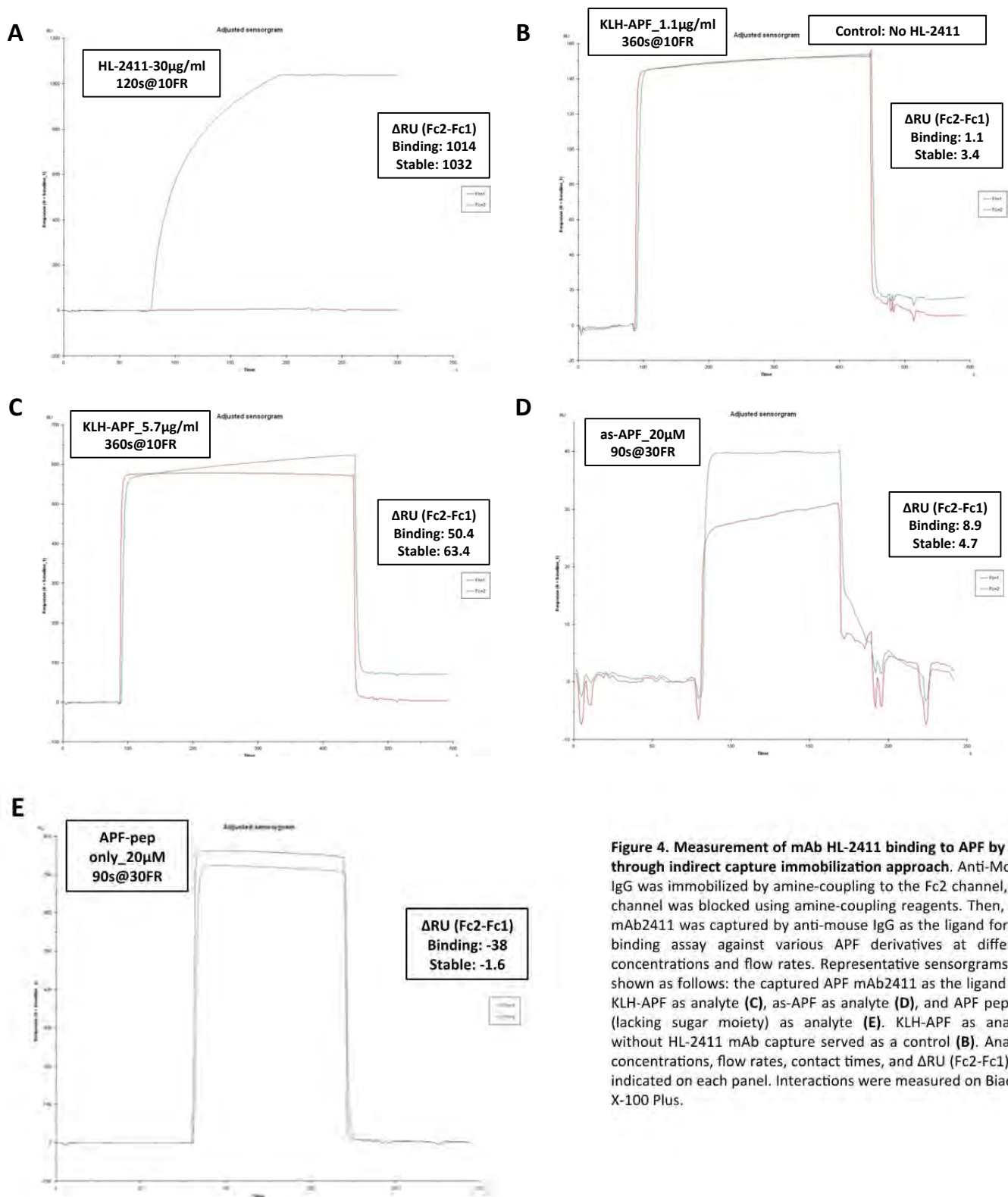


Figure 4. Measurement of mAb HL-2411 binding to APF by SPR through indirect capture immobilization approach. Anti-Mouse IgG was immobilized by amine-coupling to the Fc2 channel, Fc1 channel was blocked using amine-coupling reagents. Then, APF mAb2411 was captured by anti-mouse IgG as the ligand for the binding assay against various APF derivatives at different concentrations and flow rates. Representative sensorgrams are shown as follows: the captured APF mAb2411 as the ligand (**A**), KLH-APF as analyte (**C**), as-APF as analyte (**D**), and APF peptide (lacking sugar moiety) as analyte (**E**). KLH-APF as analyte without HL-2411 mAb capture served as a control (**B**). Analyte concentrations, flow rates, contact times, and ΔRU (Fc2-Fc1) are indicated on each panel. Interactions were measured on Biacore X-100 Plus.

To more fully characterize these antibodies for APF binding specificity, we used a Simple Western assay system (Figure 5). Electrophoresis was performed using three different APF peptides: APF synthesized by NEP (New England Peptides) at 200uM, as-APF synthesized by NEP at 200uM, and as-APF synthesized by Peptides International at 200uM) each diluted 30-fold with electrophoresis sample buffer. After electrophoresis, the Western blot was performed in situ according to the

manufacturer's protocol. Our five different APF mAbs were tested and the corresponding ECL image is shown in Figure 5. Our results indicate that mAbs HL-2409b, HL-2411, and HL-2412 generated positive Western blot bands against both APF and as-APF (active form of APF) peptides, indicating that they are specific enough to detect APF; however, mAbs 2408 and 2409a did not show a positive reaction with the APF and as-APF peptides. Interestingly, the three mAbs that bound specifically to APF are IgG1 monoclonal antibodies, whereas antibodies 2408 and 2409a are IgG3 monoclonal antibodies. Thus, we determined that HL-2409b, HL-2411, and HL-2412 show the most promise for further development and utilization in the SPR assay. The different positions of APF and as-APF bands within the SimpleWestern electrophoresis matrix may be due to various aggregated forms of APF induced by its chemical property as a glycosylated peptide.

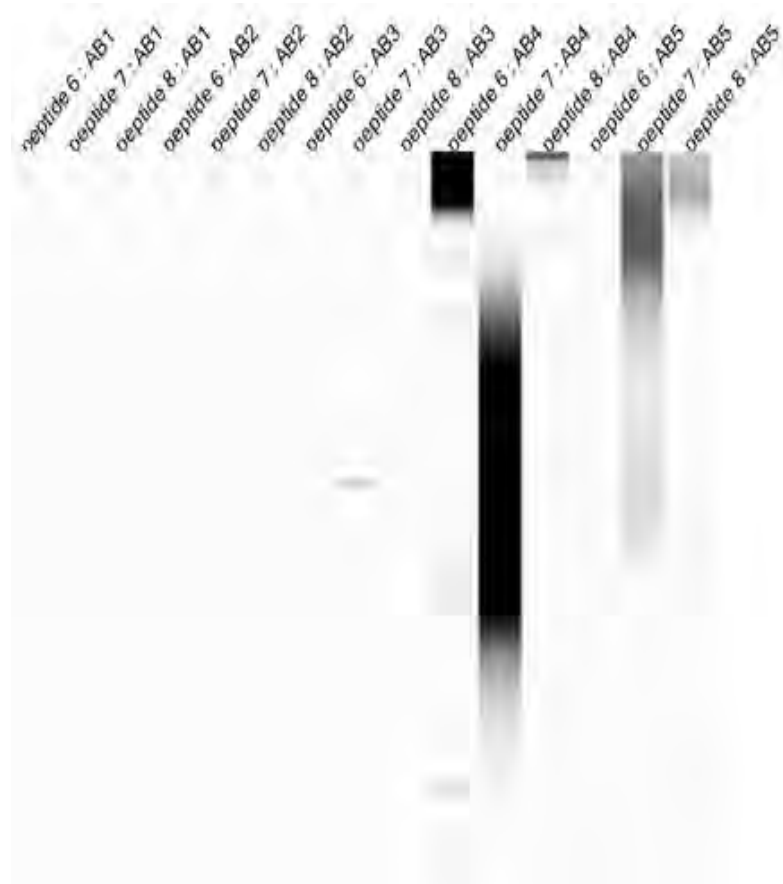
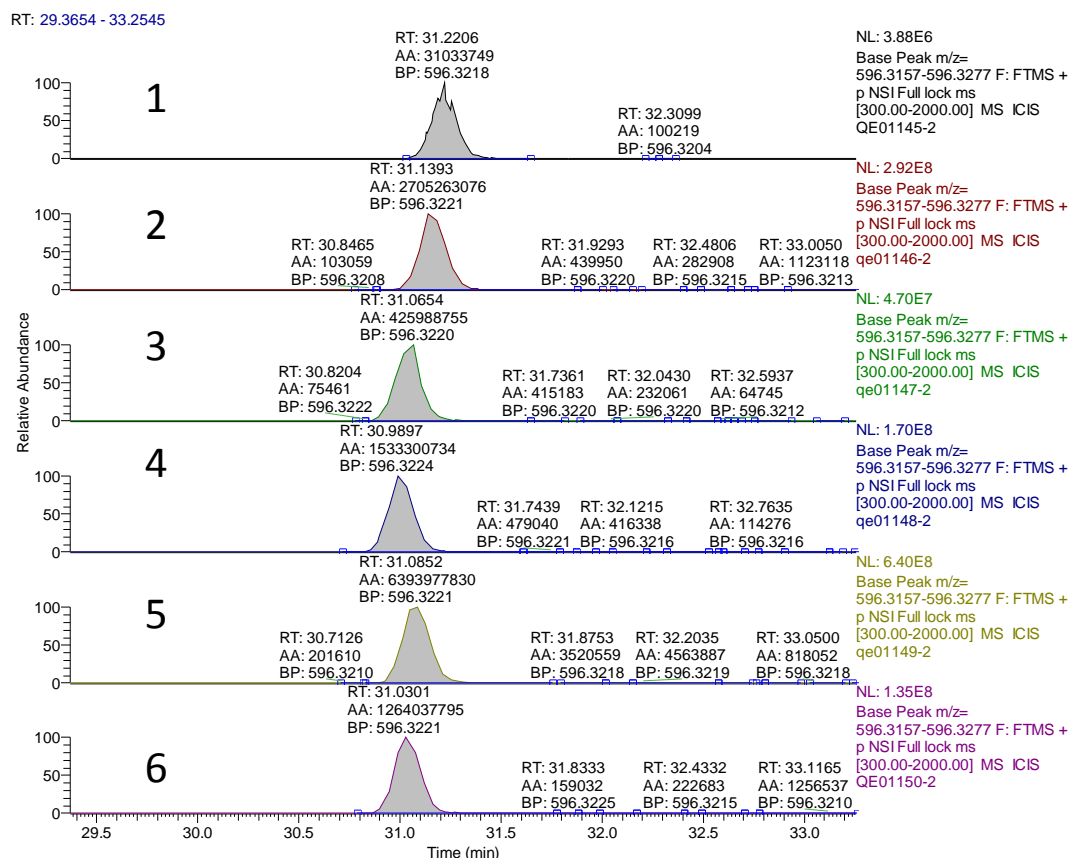


Figure 5. Detection of APF peptides by APF monoclonal antibodies using a Simple Western assay system. Three different APF peptides (peptide 6: APF synthesized by NEP at 200uM; peptide 7: as-APF synthesized by NEP at 200uM; peptide 8: as-APF synthesized by Peptide International at 200uM) were diluted 30-fold with electrophoresis sample buffer. After denaturation at 95°C for 5 min, 4ul of each sample was loaded into each capillary tube for electrophoresis by Simple Western (ProteinSimple). After electrophoresis, the Western blot was performed in situ according to the manufacturer's protocol. Five different APF antibodies were tested and the corresponding ECL image is shown. AB1=HL-2408; AB2=HL-2409a; AB3=HL-2409b; AB4=HL-2411; AB5=HL-2412

We also evaluated whether these antibodies could bind to APF using a beads based approach followed by liquid chromatography/mass spectroscopy (LC-MS) for APF identification. Using this experimental approach, we confirmed that all of the antibodies were able to capture APF, although HL-2411 bound APF most robustly, resulting in an approximately 10-fold increase in the amount of APF captured (Figure 6).

A.



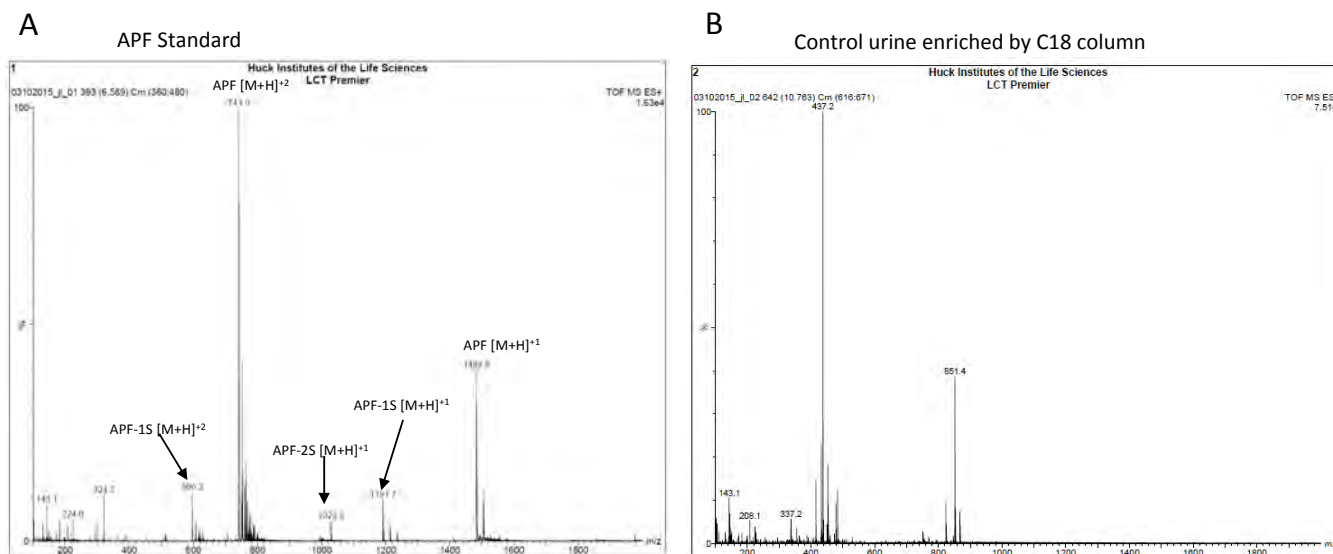
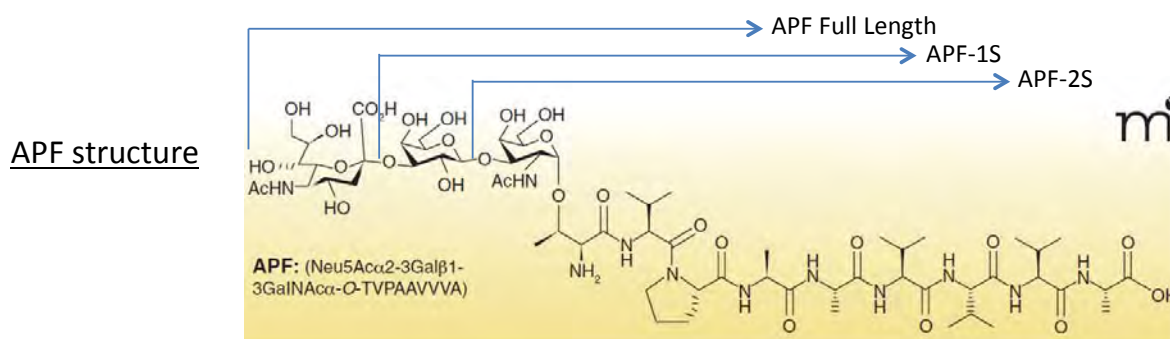
B.

| sample | peak area for 596.3221 | concentration (estimate in nM) |
|-----------------|------------------------|--------------------------------|
| Control + asAPF | 31033749 | 78.85 |
| 2408 + asAPF | 2705263076 | 346.28 |
| 2409a + asAPF | 425988755 | 118.35 |
| 2409b + asAPF | 1533300734 | 229.08 |
| 2411 + asAPF | 6393977830 | 715.15 |
| 2412 + asAPF | 1264037795 | 202.15 |

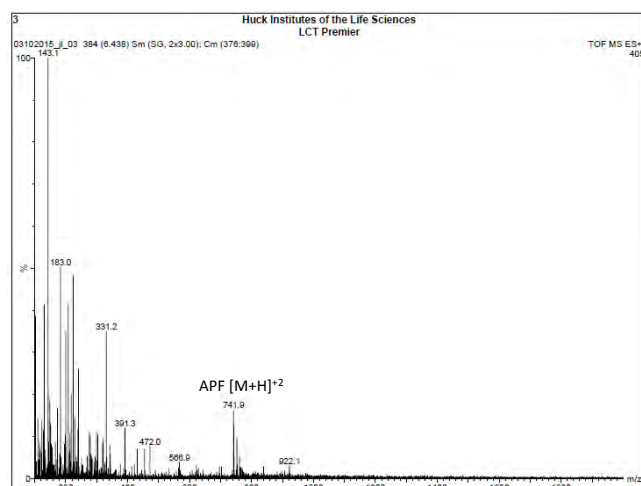
Figure 6. **Quantitation of APF in the antibody bead-based assay by LC-MS.** Purified monoclonal antibodies were immobilized to His tag affinity beads; the control was beads only without antibody. After immobilization for 1 hour and washing with PBS buffer three times, the beads were incubated with 40 μ M of APF peptide for 1.5 hour. Following three washes with PBS, the bound APF was eluted using 175 mM imidazole. The eluates were analyzed by LC-MS. The HPLC peaks and MS analysis results are shown in panel A. Panel B is the quantitated APF amount based on the LC-MS results.

1b: Optimization of conditions for sensor chip surface regeneration and binding reproducibility (months 1-12). Task 1b under Aim 1 has been accomplished as we have determined amine-coupling to be the optimal method for CKAP4 biosensor immobilization and indirect capture to be the optimal method for APF antibody biosensor immobilization.

1c: Characterization of the SPR-based assay using synthetic APF (months 12-18). We expect that this task will be accomplished within the next few months. Previously, we characterized the APF binding potential of four CKAP4 deletion mutant constructs and determined that CKAP4₁₂₇₋₃₆₀ the highest potential to detect and quantitate APF in urine samples. We have since focused our efforts on determining the optimal strategy for APF enrichment in patient urine samples in order to validate the sensitivity of detection of APF in urine sample by SPR. As shown in Figure 7, we used mass spectroscopy (MS) to determine the optimal enrichment strategy for APF from IC urine samples. Control urine was spiked with synthetic APF (40uM), followed by enrichment with C18 reverse phase resin only and combined with Jacalin oligosaccharide affinity resin. As compared to the standard APF mass spectra (Panel A), APF was enriched and detected by MS from the control urine spiked with APF (Panel C and D), but not from control urine only (Panel B). In addition, only full length APF was detected in the enriched APF from urine samples.



C Control urine + APF, enriched by C18 column



D Control urine + APF, enriched by Jacalin affinity resin and C18 column

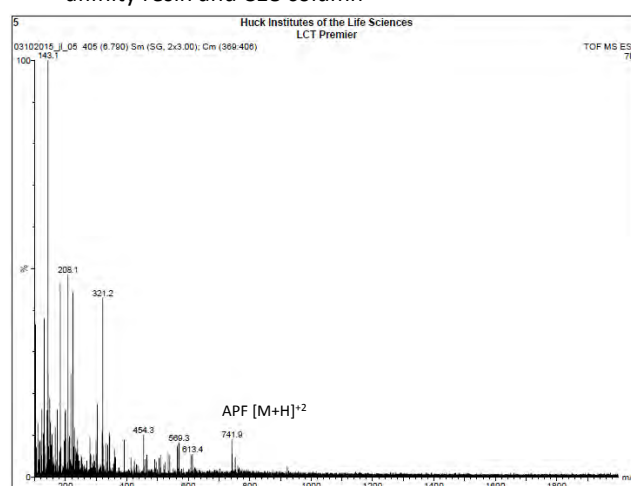


Figure 7. Confirmation of APF purified from urine sample by mass spectrometry (MS).

In order to validate the sensitivity of detection of APF in urine sample by SPR, a MS experiment was done to determine the best method to enrich APF from IC urine samples. In this experiment, control urine was charged or spiked by synthetic APF (40uM), followed by enrichment with C18 reverse phase resin only and combined with Jacalin oligosaccharide affinity resin. After the enrichment of APF from 120ul of control urine and several repeated washes, the eluted peptides were dried by lyophilization. The total peptides were then dissolved in water for analysis by Waters LCT Premier time-of-flight mass spectrometer. The mass spectra are shown in panels A through D with the identified APF peaks indicated. As compared to the standard APF mass spectra (Panel A), APF was enriched and detected by MS from the control urine charged with APF (Panel C and D), but not from control urine only (Panel B). In addition, only full length APF was detected in the enriched APF from urine samples. Testing of endogenous APF from IC patient urine is under investigation.

Using this urine enrichment strategy, we tested the dynamic range and sensitivity of the SPR-based assay employing the CKAP4₁₂₇₋₃₆₀ mutant as the immobilized biosensor. For this experiment, we used urine from healthy donors spiked with as-APF as the mimetic for IC patient urine and CKAP4₁₂₇₋₃₆₀ as the ligand, since it yielded the highest binding affinity as observed earlier. Various dilutions of a control urine sample spiked with APF were prepared and injected over a CM5 chip surface pre-treated with the CKAP4₁₂₇₋₃₆₀ ligand (Fc2 channel). To account for background interference from urinary constituents, corresponding dilutions of unspiked control urine were also run to serve as individual controls. The representative sensorgrams (Figure 8) show that it is possible to detect low concentrations of APF in urine using our SPR-based assay. Additional experiments using spiked urine from healthy donors will be undertaken to accurately establish detection limits for this assay. With further refinements, we expect that it will be possible to detect sub-micromolar concentrations of urinary as-APF/APF.

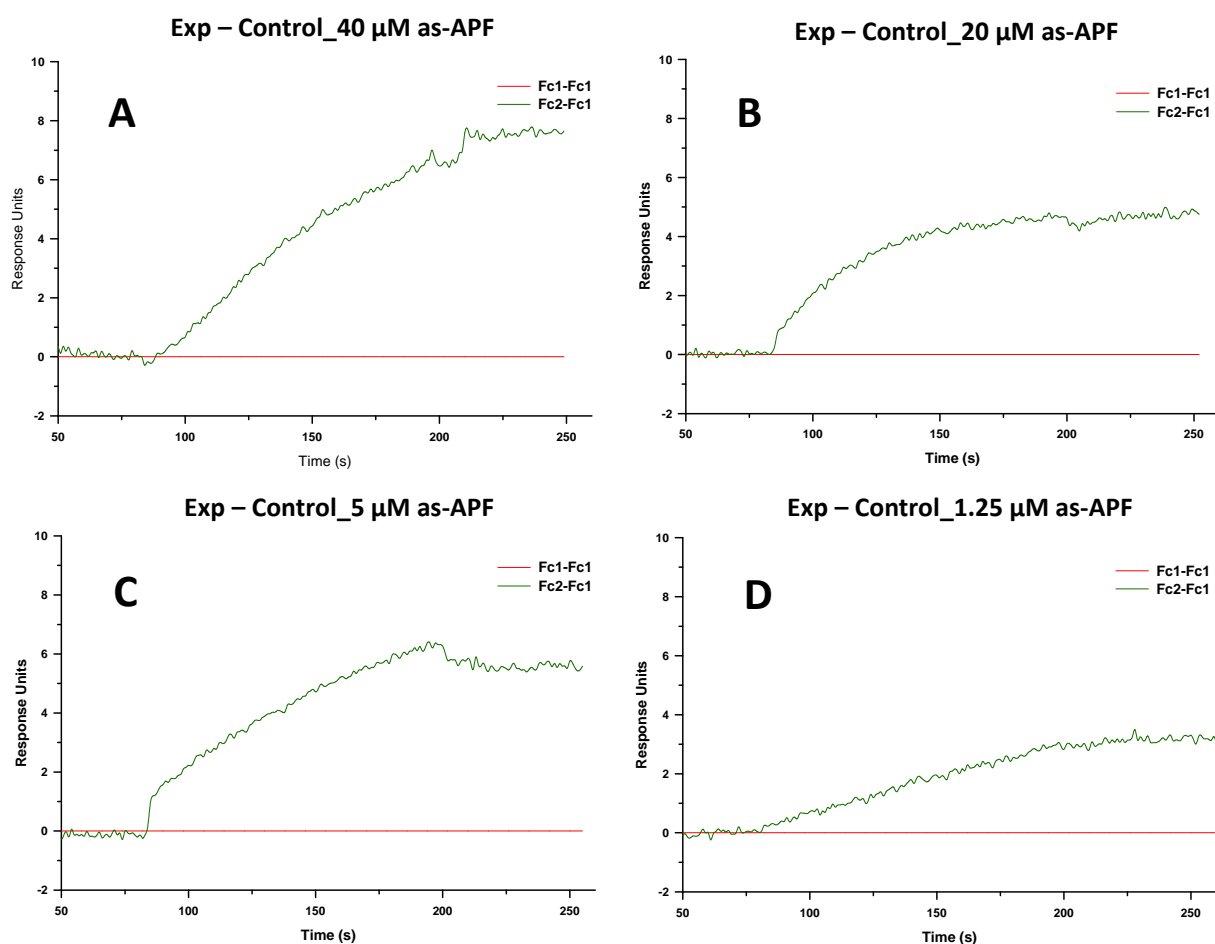


Figure 8. Detection of synthetic as-APF in spiked control urine from healthy donors. Control urine was mixed 1:1 with as-APF stock (200 μ M) solution. Various dilutions of this mixture in SPR running buffer were prepared and injected over a CM5 chip surface immobilized with CKAP4₁₂₇₋₃₆₀ by amine-coupling on the Fc2 channel. Fc1 channel was similarly treated but without CKAP4, as the control. Final concentrations of as-APF were 40 μ M (**A**), 20 μ M (**B**), 5 μ M (**C**), and 1.25 μ M (**D**). Resulting sensorgrams were corrected for background interference from the urine, and re-plotted after subtracting the RU trace from the control (Fc1) channel.

Goals in Aim 1 that have not yet been met include full characterization of 20 samples containing various levels of synthetic APF spiked in urine from healthy donors as a first test of the system's diagnostic ability. To date, we have tested 5 spiked urine samples using a Jacalin affinity resin and/or C18 column enrichment strategy to detect APF by SPR, but would like to fully optimize our enrichment procedure using the spiked control urine samples prior to using the actual urine specimens collected as part of the study.

SOW – Specific Aim 2/Task 2: *Determine the ability of the SPR-based assay to detect APF in urine from patients with IC (months 1-36).* During this annual reporting period, effort was expended primarily on Tasks 2a, 2b, and 2c under Specific Aim 2. We have made substantial progress in year 2 as detailed below.

2a: Regulatory review and approval by Institutional Review Boards and DoD Human Research Protection Office (months 1-3): This has been accomplished and approval has been received by all regulatory agencies.

2b: Recruitment of human subjects for urine sample acquisition (months 4-24): The recruitment of human subjects for urine sample acquisition is nearly complete at the University of Pennsylvania. To date, 44 patients have been recruited and consented; 10 of these patients no longer want to participate; 4 have consented but have not yet provided a urine specimen; and 29 urine samples have been collected and processed. With patient follow-up appointments pending in early December, we expect that urine specimen collection at the University of Pennsylvania will be completed by December 2015. Recruitment of human subjects at the Commonwealth Medical College is complete with 30 control subjects recruited and urine samples collected and processed. In order to age match the newly consented IC patients at Penn who have not yet provided a urine specimen, an additional 3-7 control patients may need to be recruited at TCMC. We expect to have this resolved within the next few weeks and will contact our Institutional Review Board should the need arise to increase enrollment.

2c: Testing of biological urine specimens by the cellular proliferation assay (months 4-30). Testing of biological urine specimens by the cellular proliferation assay is underway at the University of Maryland. To date, Dr. Keay's lab has received and blindly tested 25 urine specimens. She will be receiving the next shipment of specimens in three weeks. We expect that this task will be accomplished in the next 3-5 months of the grant.

2d: Testing of biological urine specimens by the SPR-based assay and comparison of results with cellular proliferation assay results (months 24-36). We expect that this task will be accomplished within the final year of the grant once the assay is fully characterized and optimized and Dr. Keay has completed her testing.

2e: Statistical analysis (months 24-36). We expect that this task will be accomplished in the final year of the grant once testing is completed.

Goals in Aim 2 that have not yet been met include the completion of human subject recruitment and urine sample collection; testing of the biological urine specimens by the cellular proliferation assay and the SPR-based assay; and the final statistical comparison of the results. We expect that these objectives will be completed within the final year of the contract period.

Provided Opportunities for Training and Professional Development

An abstract titled "*Development of a surface plasmon resonance-based assay to detect antiproliferative factor in interstitial cystitis patient urine*" and authored by Chavda B, Ling J, and Planey SL was presented at the American Society for Molecular Biology and Biochemistry Annual Meeting in Boston, MA on 29-Mar-2015. This provided a professional development opportunity for the post-doctoral fellow supported by this project, Burzin Chavda, PhD, to present our findings at a national conference. We also submitted an abstract which was accepted as a poster for the 2015 Military Health System Research Symposium (MHSRS) on 01-Jun-2015. Dr. Planey attended the MHSRS conference on August 17-20 and presented the teams findings.

Dissemination of Results to Communities of Interest

Nothing to Report

Plan for Accomplishing Goals Next Reporting Period

Our goals for the next quarter under Aim 1 is to continue characterization of the SPR-based assay using the CKAP4₁₂₇₋₃₆₀ biosensor and synthetic APF spiked in urine from health donors in order to determine the dynamic range and sensitivity of the SPR-based assay following APF enrichment from urine. We will concomitantly test the HL-2409b, HL-2411, and HL-2412 antibodies as biosensors in the SPR-based assay to determine which approach has the greatest sensitivity and dynamic range for final testing. Under Aim 2, we plan to complete collection and acquisition of all patient urine specimens by December, so that future testing and analysis can be completed within the final year of the contract period.

4. IMPACT:

Impact on the Development of the Principal Discipline(s) of the Project

The biophysical interaction of APF with its cellular receptor, CKAP4, has not been previously characterized. The results of our current study demonstrate direct binding of CKAP4 to APF and/or as-APF with real time kinetics, providing an important tool to characterize how the two interact specifically to elicit APF's pathological effects. As shown in Figure 1 and Table 1 of this report, regions of the CKAP4 N-terminal, extracellular domain are required for binding APF and as-APF. Table 1 lists the parameters of the binding kinetics for the APF interaction with the CKAP4 extracellular domain mutants using a 1:1 binding kinetics model. Importantly, both CKAP4₁₂₇₋₃₆₀ and CKAP4₃₆₁₋₅₂₄ exhibited a fast association constant (k_{on}) and a slow dissociation constant (k_{off}), therefore yielding high binding affinity ($K_D = k_{off}/k_{on}$; K_D for CKAP4₁₂₇₋₃₆₀: 1.34 μ M, and K_D for CKAP4₃₆₁₋₅₂₄: 2.27 μ M), which fits the general model of strong interaction and suggests that the primary APF binding site is located within the Aa 127-524 region of the CKAP4 extracellular domain. Thus, we determined that the CKAP4₁₂₇₋₃₆₀ and CKAP4₃₆₁₋₅₂₄ mutants exhibit improved binding to APF with normal proportional response to increasing dose of ligand and the maximum binding response (Rmax), making a robust SPR-based assay feasible. Further, these data have revealed a novel APF binding site, suggesting that targeting this region of CKAP4 to inhibit APF binding may be a useful strategy for treating IC related symptoms.

What was the Impact on other Discipline(s)

We expect that with additional refinement over the next reporting period, the SPR-based assay will overcome current barriers associated with validation of APF as a diagnostic biomarker for IC by being able to specifically detect and measure APF levels in urine. Thus, this assay represents a tangible product that could be developed into an affirmative diagnostic test for IC with the advantages of being rapid, repeatable, and non-invasive. This would potentially impact clinical practice by obviating the need for more costly invasive procedures such as cystoscopy and hydrodistension and curtail rates of misdiagnosis or improper treatment of patients who have symptomological overlap with IC. Accurate and earlier diagnosis would improve patient care and quality of life leading to better management of comorbidities and reduce healthcare costs.

What was the Impact on Technology Transfer

Nothing to Report

What was the Impact on Society Beyond Science and Technology

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in Approach and Reasons for Change

Nothing to report

Actual or Anticipated Problems or Delays and Actions or Plans to Resolve Them

During this annual reporting period, we encountered a problem with our supply of synthetic APF peptide, which we were using for characterization of the SPR-based assay. We used a new vendor to synthesize the peptide; however, we observed differences in its binding characteristics by SPR. To resolve the issue, we contacted our original supplier to provide additional APF peptide. Despite a delay, they were able to synthesize more peptide for us. We are also ordering the peptide more regularly and in larger quantities, so as to avoid running out of stock in the future.

Changes that had a significant impact on expenditures

Nothing to report

Significant Changes in Use or Care of Human Subjects

To date, 44 patients have been recruited and consented at the University of Pennsylvania; 10 of these patients no longer want to participate; 4 have consented but have not yet provided a urine specimen;

and 29 urine samples have been collected and processed. With patient follow-up appointments pending in early December, we expect that urine specimen collection at the University of Pennsylvania will be completed by December 2015. Recruitment of human subjects at the Commonwealth Medical College is complete with 30 control subjects recruited and urine samples collected and processed. However, in order to age match the newly consented IC patients at Penn who have not yet provided a urine specimen, an additional 3-7 control patients may need to be recruited at TCMC. Should the need arise to increase enrollment at TCMC, we will seek approval from our Institutional Review Board and report this to the agency.

6. PRODUCTS:

Publications, Conference Papers, and Presentations

a. Journal Publications:

- Chavda B, Majernick T, Ling J, Planey SL. APF binding to cytoskeleton associated protein 4 requires sites within its N-terminal extracellular domain. *Journal of Biological Chemistry*, In preparation, 2015.

b. Abstracts/Presentations:

- “Development of a surface plasmon resonance-based assay to detect antiproliferative factor in interstitial cystitis patient urine” and authored by Chavda B, Ling J, and Planey SL was presented at the American Society for Molecular Biology and Biochemistry Annual Meeting in Boston, MA on 29-Mar-2015.
- “Development of a surface plasmon resonance-based assay to detect antiproliferative factor in interstitial cystitis patient urine” and authored by Chavda B, Majernick T, Ling J, and Planey SL was accepted as a poster for the 2015 Military Health System Research Symposium (MHSRS) in Ft. Lauderdale, Florida and presented on 18-Aug-2015.

Website(s) or other Internet site(s)

Nothing to Report

Technologies or Techniques

Nothing to Report

Inventions, Patent Applications, and/or Licenses

Non-provisional Patents:

1. US Patent Number 8,962,341 B2 “Cell-Based Detection of APF Through Its Interaction with CKAP4 for Screening of Interstitial Cystitis” Inventors: Sonia Lobo Planey and David A. Zacharias issued on 24-Feb-2015.
2. US Patent No. 13/911,242 “Cell-Based Detection of APF Through Its Interaction with CKAP4 for Diagnosis of Interstitial Cystitis” Inventors: Sonia Lobo Planey and David A. Zacharias issued on 07-Jul-2015.

Other Products

An alternative approach taken to increase the sensitivity of the SPR assay has resulted in the production of five purified monoclonal antibodies that display specific activity against APF-KLH in a dot blot assay and specific binding to the active form of APF by the SPR assay. These antibodies are being further characterized and may offer a new approach and/or research tool to specifically measure APF in urine samples.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

Participating Individuals

| Personnel | Role in Project | Current Status | Percent Effort |
|------------------------|------------------------|---|----------------|
| Sonia Lobo Planey, PhD | Principal Investigator | No change since previous quarterly report | 40 |
| Jun Ling, PhD | Collaborator | No change since previous quarterly report | 15 |
| Betsy Meade, RN, BSN | Research Associate | No change since previous quarterly report | 5 |
| Burzin Chavda, PhD | Post-Doc | No change since previous quarterly report | 100 |
| Thomas Majernick, MS | Research Technician | No change since previous quarterly report | 100 |

Changes in Active Other Support of the PD/PI(s) or Senior/Key Personnel Since the Last Reporting Period

Nothing to Report

Partnering Organizations

Organization Name: Hospital of the University of Pennsylvania

Location of Organization: Philadelphia, Pennsylvania

Partner's contribution to the project: Facilities and collaboration.

We are collaborating with Dr. Phillip Hanno and his health care team to recruit IC patients for this study during routine office visits to the Penn Urology Clinic at HUP for the management of IC as well as with the retrieval of patient information for use in future correlation analysis.

Organization Name: University of Maryland

Location of Organization: Baltimore, Maryland

Partner's contribution to the project: Facilities and collaboration.

We are collaborating with Dr. Keay and individuals in her laboratory to blindly test biological urine specimens by the cellular proliferation assay. Dr. Keay will send her results to us for comparison with the SPR assay results and will assist in the interpretation of the final analysis.

8. SPECIAL REPORTING REQUIREMENTS:

Quad Chart

9. APPENDICES:

References

REFERENCES:

1. Sant, G.R. and P.M. Hanno, *Interstitial cystitis: current issues and controversies in diagnosis*. Urology, 2001. 57(6 Suppl 1): p. 82-8.
2. Payne CK, Joyce GF, Wise M, Clemens JQ. *Interstitial cystitis and painful bladder syndrome*. J Urol 2007;177:2042-9.
3. Clemens, J.Q., et al., *Prevalence of interstitial cystitis symptoms in a managed care population*. J Urol, 2005. 174(2): p. 576-80.
4. Clemens, J.Q., et al., *Prevalence and incidence of interstitial cystitis in a managed care population*. J Urol, 2005. 173(1): p. 98-102; discussion 102.
5. Curhan, G.C., et al., *Epidemiology of interstitial cystitis: a population based study*. J Urol, 1999. 161(2): p. 549-52.
6. Leppilahti, M., et al., *Prevalence of clinically confirmed interstitial cystitis in women: a population based study in Finland*. J Urol, 2005. 174(2): p. 581-3.
7. Keay S, Zhang CO, Shoenfelt JL, Chai TC. Decreased in vitro proliferation of bladder epithelial cells from patients with interstitial cystitis. Urology 2003;61:1278-84.
8. Keay SK, Zhang CO, Shoenfelt J, et al. Sensitivity and specificity of antiproliferative factor, heparin binding epidermal growth factor-like growth factor, and epidermal growth factor as urine markers for interstitial cystitis. Urology 2001;57:9-14.
9. Keay S. Cell signaling in interstitial cystitis/painful bladder syndrome. Cell Signal 2008;20:2174-9.

Validation of APF as a Urinary Biomarker for Interstitial Cystitis

PR121048 - Investigator-Initiated Research Award

W81XWH-13-1-0454



PI: Sonia Lobo Planey, Ph.D.

Org: The Commonwealth Medical College

Award Amount: \$945,142.00

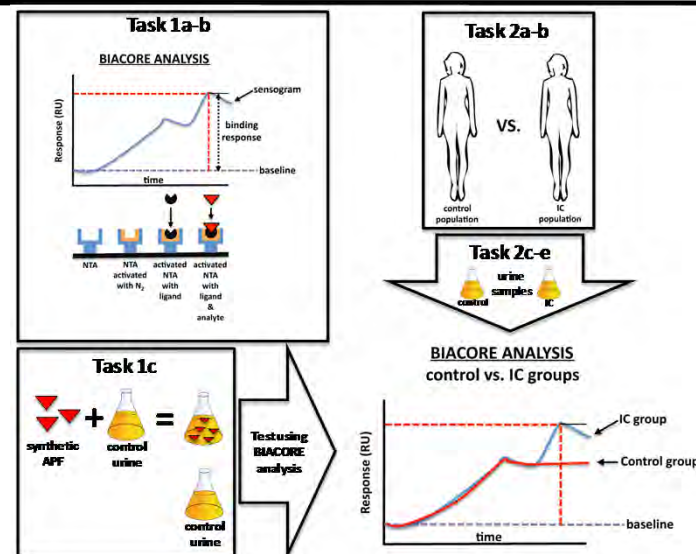
Study Aim(s)

Aim 1: To develop and characterize a SPR-based assay employing a CKAP4 immobilized biosensor to detect APF.

Aim 2: Determine the ability of the SPR-based assay to detect APF in urine from patients with IC.

Approach

To accomplish Aim 1 we will optimize rCKAP4 activity and sensor chip immobilization to achieve maximal CKAP4/APF binding efficiency and reproducibility. We will then determine linearity and range of the assay using known concentrations of synthetic APF spiked in urine from healthy donors. In Aim 2 we will determine the ability of the SPR-based assay to detect and quantitate APF in urine samples from patients with IC (45) versus non-IC, age-matched controls (45). Sensitivity and specificity of the SPR assay to detect APF will be determined by comparison with cellular proliferation assay results obtained by Dr. Keay. Statistical analysis will be performed to assess APF's utility as a diagnostic and/or prognostic biomarker for IC.



SPR assay characterization using a CKAP4₁₂₇₋₃₆₀ biosensor and synthetic APF spiked in urine.

Timeline and Cost

| Activities | CY | 13 | 14 | 15 | 16 |
|--|----|-------------|--------------|--------------|--------------|
| Task 1a-b. Improved CKAP4/APF binding efficiency and reproducibility | | | | | |
| Task 1c. Characterization of SPR-based assay using synthetic APF | | | | | |
| Task 2a-b. Subject recruitment and acquisition of 90 urine specimens | | | | | |
| Task 2c-e. Sample testing by SPR assay and validation of APF | | | | | |
| Estimated Budget (\$K) | | \$50 | \$323 | \$313 | \$259 |

Goals/Milestones

CY13 Goal – SPR assay development

☒ Optimization of rCKAP4 activity and immobilization

CY14 Goals – SPR assay characterization

☒ Improved CKAP4/APF binding efficiency and reproducibility

☐ Characterization of 20 samples using synthetic APF

CY15 Goals – Acquisition and testing of 90 biological specimens

☐ Testing for APF activity by cellular proliferation assay

☐ Testing for APF by SPR-assay

CY16 Goals – Statistical analysis and comparison of assay results

☐ First direct measurement of APF in human urine

☐ Assess APF's utility as a diagnostic biomarker for IC

Comments/Challenges/Issues/Concerns

• No concerns at this time with the scientific goals/accomplishments

Budget Expenditure to Date

Projected Expenditure: \$636,032

Actual Expenditure: \$568,885

Updated: (September, 2015)